Original Article

A validated RP-HPLC-UV method for quantitative determination of puerarin in *Pueraria tuberosa* DC tuber extract

Abstract

Background: *Pueraria tuberosa* (Fabaceae) is a well-known medicinal herbs used in Indian traditional medicines. The puerarin is one of the most important bioactive constituent found in the tubers of this plant. Quantitative estimation of bioactive molecules is essential for the purpose of quality control and dose determination of herbal medicines. The study was designed to develop a validated reversed phase high-performance liquid chromatography (RP-HPLC) method for the quantification of puerarin in the tuber extract of *P. tuberosa.* **Materials and Methods:** The RP-HPLC system with Luna C18 (2) 100 Å, 250 × 4.6 mm column was used in this study. The analysis was performed using the mobile phase: 0.1% acetic acid in acetonitrile and 0.1% acetic acid in water (90:10, v/v) under column temperature 25°C. The detection wavelength was set at 254 nm with a flow rate of 1 ml/min. The method validation was performed according to the guidelines of International Conference on Harmonization. **Results:** The puerarin content of *P. tuberosa* extract was found to be 9.28 ±0.09%. The calibration curve showed good linearity relationship in the range of 200-1000µg/ml (r^2>0.99). The LOD and LOQ were 57.12 and 181.26µg/ml, respectively and the average recovery of puerarin was 99.73% ±1.02%. The evaluation of system suitability, precision, robustness and ruggedness parameters were also found to produce satisfactory results. **Conclusions:** The developed method is very simple and rapid with excellent specificity, accuracy and precision which can be useful for the routine analysis and quantitative estimation of puerarin in plant extracts and formulations.

Key words: *Pueraria tuberosa*, puerarin, RP-HPLC, validation

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INTRODUCTION

Pueraria tuberosa DC (Family: Fabaceae) is a reputed medicinal herb of Indian traditional system of medicines distributed throughout tropical parts of the India.[1] In India it has several vernacular names like in *Bengali*: shimia, batraji, *Gujarati*: vidarikand; *Hindi*: bilaikand; *Kannada*: gumadi gida; *Malayalam*: mutukku; *Marathi*: badra; *Tamil* and *Telugu*: dari gummadi.[2] Traditionally, the *P. tuberosa* tubers (PTT) are used as tonic, anti-rheumatic, diuretic, galactogogue, vital energy and immune booster.[1,3] PTT was reported to contain puerarin, daidzein, puerarone, coumestan, tuberosin, pterocarpintuberosin, puetuberosanol and hydroxytuberosone.[4] The isoflavone, puerarin [Figure 1], one of the most active constituent of PTT, revealed wide range of pharmacological activities including hypoglycemic,[5] anti-cancer,[6] cardioprotective,[7] neuroprotective,[8] anti-allergic,[9] and anti-arrhythmic[10] activity.

The quantitative determination of bioactive metabolites in the extracts and formulations is essential for quality control and dose determination of herbal medicines.[11] Several analytical methods include high-performance thin-layer chromatography,[12] high-performance liquid chromatography,[13] high-performance capillary electrophoresis,[14] high-performance liquid chromatography mass spectrometry[15] and fluorescence spectrometry[16] have been reported for the identification and quantification of puerarin. To best of our knowledge, there is only one report dealing with the quantitative estimation

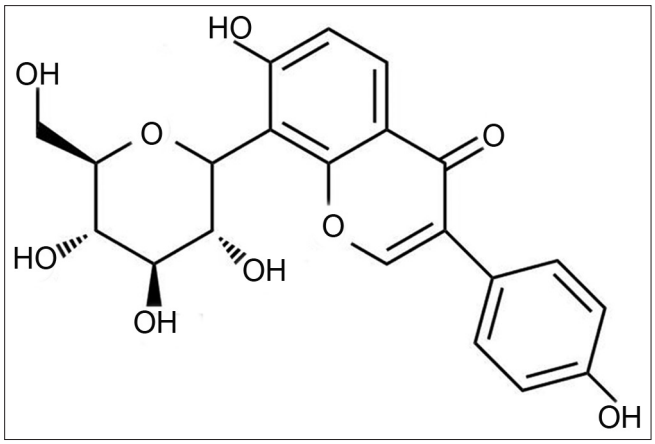

Figure 1: Chemical structure of puerarin

of puerarin in the PTT extract but no data has been given about the validation characteristics of the method used.[17] Thus, the present study was designed to develop a validated RP-HPLC method for the quantification of puerarin in PTT extract.

MATERIALS AND METHODS

Chemicals and reagents

The standard puerarin (purity 91.5%) was procured from LGC Promochem Pvt. Ltd., Bangalore, India and all other solvents (HPLC grade) were purchased from Merck (Mumbai, India). All the solvents were filtered through membrane filters of 0.45 mm pore size (Millipore) before use.

Instrumentation

The HPLC system (Waters, Milford, MA, USA) was consisted of a 600 controller pump, a multiple-wavelength ultraviolet-visible (UV-Vis) detector, an in-line AF 2489 series degasser, a rheodyne 7725i injector with a 20 ml loop with integrated Empower2 integration software. The separation was performed using Luna C18 (2) 100 Å, 250 × 4.6 mm filled with 5 µm particles (Phenomenex, Torrance, CA, USA) column.

Chromatographic conditions

The assay of puerarin was performed using externally standardized isocratic conditions. The separation was carried out using the mobile phase (pH 7.0) consisted of 0.1% acetic acid in acetonitrile and 0.1% acetic acid in water (90:10, v/v) which was degassed and filtered before run the column. The column temperature was maintained at 25°C and each injection volume was 20 µl. The wavelength was set at 254 nm with a flow rate of 1 ml/min and the run time was set at 20 min. The peak identification was performed by comparison of the retention time (RT) of the reference standard with the extract.

Plant material and extraction

PTT were procured from the local vendor and the sample was authenticated from the Department of Botany and Forestry, Vidyasagar University, India. The voucher specimen (VU/BOT/DB/17/12) has been deposited at the herbarium of the Department of Botany and Forestry, Vidyasagar University, India.

The air dried (20-25°C) plant material (500 g) was extracted with 70% ethanol by cold maceration process for 15 days at 25°C. The extract was filtered through Whatman filter paper No. 1, pore size (11 µm) and dried through rotary evaporation followed by lyophilization.

Sample and standard preparation

Crude extract (100 mg) and puerarin (4 mg) were dissolved in mobile phase solution to prepare 1 mg/ml solutions of extract and standard. The standard solution was subsequently diluted to prepared different concentrations (200-1000 µg/ml) of standard solutions. All aliquots were filtered through Whatman's syringe filters (NYL 0.45 µm) before analysis.

Calibration curve

The calibration curve was established by analyzing the different concentrations of puerarin standard solution ranging from 200-1000 µg/ml. The calibration curve was constructed by linear regression analysis of the peak area against the respective concentration of puerarin. The quantification of puerarin in the extract was quantified in reference to this calibration curve.

Method validation

The RP-HPLC method was validated for system suitability, specificity, limits of detection and quantification, accuracy, precision, robustness and ruggedness. The method validation was performed according to the recommended guidelines of International Conference on Harmonization (ICH).[18]

System suitability

The system suitability test was carried out to establish the parameters such as percentage relative standard deviations (% RSD) for RT, peak area response, tailing factor, theoretical plates, resolution factor and capacity factor. The test was performed by analysing six replicates (n = 6) of a reference standard solution (200 µg/ml) and the % RSD of the parameters was calculated.

Specificity

The method specificity was evaluated to minimize errors due to the presence of any other compounds. The method specificity was assessed by analyzing the chromatogram of standard puerarin and extract for peak purity. The peak purity of puerarin was determined using multivariate analysis by comparison of RT and peak area.

Limits of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ were assessed by determining the standard deviation (σ) of the response and the slope (*S*) of the linear equation. The following formulas were used to determine the LOD and LOQ:[18]

LOD =3.3 σ/*S*

LOQ =10 σ/*S*

Where, σ = standard deviation of the response from the number of blank run and *S* = slope of the calibration curve.

Accuracy

To ensure the accuracy of the analytical method, the recovery study of reference standard in the test sample was performed. The method was employed the addition of known quantities of reference standard with the pre-analysed extract sample (n = 3) followed by the re-analysis of the contents by the proposed method. The recovery of the standard was expressed as % RSD from mean recovery of the each theoretical concentration.

Precision

Precisions of the method were evaluated by analysing the extract and different concentrations (200-1000 µg/ml) of reference standard six times on the same day for intra-day and on six successive days (n = 6) for inter-day precision. The mean and % RSD was calculated for intra-day and inter-day runs.

Robustness

Robustness study was carried out by analyzing the standard solution (200 µg/ml) under critical modifications of optimum conditions set for this method. The standard solution was analyzed with the small changes in the mobile phase ratio, flow rate, detection wavelength, pH, and column temperature to determine their effect on the RT, peak area response and recovery. The % RSD of RT and peak area response and percentage of mean recovery was calculated.

Ruggedness

The method ruggedness was performed by the analysis (n = 3) of different concentrations of standard solution and extract on different column along with different HPLC system. The HPLC system (Shimadzu, Tokyo, Japan) was consisted of a binary reciprocating pump with a SPD-M20A photo diode array (PDA) detector and a rheodyne 7725i injector with a 20 ml loop with integrated LC solutions software. The separation was achieved using Xterra, RP 18, 5 µm, 4.6 × 250 mm column (Waters, Milford, MA, USA). The % RSD of RT, peak area response and percentage of recovery was calculated.

Statistical analysis

The results were statistically analyzed using Graph Pad prism version 5.0. The results were calculated as the mean ± SD/SEM.

RESULTS AND DISCUSSION

The HPLC is a unique, versatile, universal and well recognized tool for qualitative and quantitative evaluation of herbal products against their respective bioactive molecules in terms of quality and batch-to-batch reproducibility.[19,20] In present RP-HPLC analysis, chromatogram of the extract showed sharp peak for puerarin at RT 5.21±0.08 min which was comparable with the standard puerarin (RT, 5.20 ±0.01 min) [Figures 2a, b]. The calibration curve of standard puerarin showed good linearity relationship in the specified concentration range (200-1000 µg/ml) with a correlation coefficient (r^2) greater than 0.99. The quantity of puerarin in the extract was found to be 9.28 ± 0.09% (w/w). The [Figure 2b], demonstrated the clear separation of puerarin with adequate peak resolution and there was no peaks at RT range of 5.21 min which indicates the method is selective for puerarin. The results of system suitability parameters were given in Table 1 and % RSD of the parameters were found to be less than 2% indicating the system suitability of the method. The LOD and LOQ for puerarin were found to be 57.12

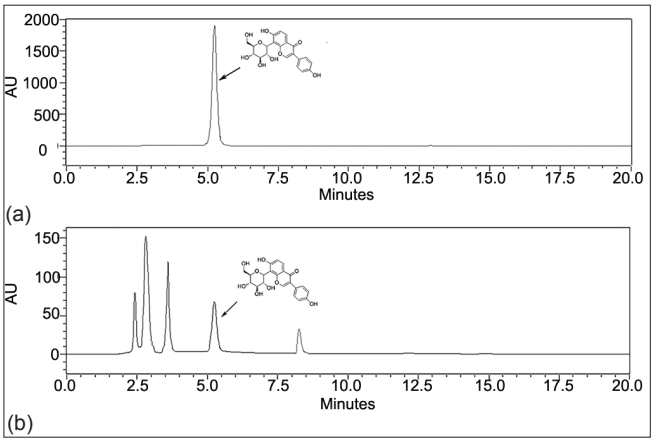

Figure 2: (a) RP-HPLC chromatogram of puerarin (b) RP-HPLC chromatogram of *P. tuberosa* extract

and 181.26 µg/ml, respectively. The mean recovery of puerarin at different concentrations was 99.73% ± 1.02% which indicated the good accuracy of this method [Table 2]. Inter-day and intra-day precision results have been represented in Table 3. The % RSD of inter- and intra-day analysis of standard and extract were found to be lower than 1.71% with a high repeatability in the RT. There was no significant difference in the inter- and intra-day analysis indicates the proposed method is very suitable for the analysis of puerarin in herbal medicines.

The results of method robustness were given in Table 4 and no significant variation in RT, peak area response and recovery of puerarin were observed under modified critical conditions and the % RSD was always less than 2% which indicates the proposed method is robust. The method ruggedness was determined by comparing the results of RT, peak area response and the assay of puerarin obtain from the different HPLC systems. The % RSD was found to be less than 2% [Table 5] which indicates the method is rugged.

Table 1: System suitability parameters for puerarin (*n* = 6)

Injection number	Retention time	Response	No. of theoretical plates	Tailing factor	Resolution factor (R_s)	Capacity factor (k′)
1	5.25	6952274.13	3223.43	1.13	4.18	4.25
2	5.27	6942338.36	3175.58	1.12	4.11	4.27
3	5.24	6931128.52	3149.66	1.11	4.15	4.24
4	5.27	6954319.91	3162.62	1.15	4.21	4.23
5	5.23	6938185.61	3132.53	1.13	4.07	4.27
6	5.26	6946491.11	3159.28	1.14	4.15	4.26
Mean	5.25	6944122.94	3167.18	1.13	4.15	4.25
% RSD	0.31	0.13	0.98	1.25	1.20	0.38

Table 2: Results of the recovery experiment from *P. tuberosa* extract (*n* = 3)

Puerarin added to extract (µg)	Total content of puerarin (µg)	Actual content of puerarin found (µg)	Recovery (%)	Mean recovery (%)
0	9.28	9.27±0.03	99.93±0.27	99.73±1.02
200	209.28	209.49±5.15	100.10±2.46	
400	409.28	407.49±2.64	99.56±0.65	
600	609.28	607.57±6.40	99.72±1.05	
800	809.28	808.33±3.65	99.88±0.45	
1000	1009.28	1001.17±3.48	99.20±0.34	

Table 3: Intra-day and inter-day precision (*n* = 6)

Amount (µg/ml)	Intra-day precision (*n* = 6)				Inter-day precision (*n* = 6)			
	Retention time (RT)		Response (AU)		Retention time (RT)		Response (AU)	
	Mean	% RSD	Mean Area	% RSD	Mean	% RSD	Mean Area	% RSD
200	5.23	0.43	6937841.50	0.60	5.24	1.28	6945837.67	0.19
400	5.20	0.27	13416009.17	0.06	5.26	0.84	13251004.50	1.18
600	5.20	0.21	20799448.50	0.07	5.24	1.08	20370758.67	1.71
800	5.23	0.53	29454254.67	0.46	5.24	1.10	29364880.50	0.54
1000	5.23	0.84	41017461.60	0.16	5.20	0.22	41034967.33	0.19
Extract	5.22	1.59	685408.06	0.24	5.24	1.38	676265.83	0.70

Table 4: Robustness of method (*n* = 3)

Parameter	Proposed	Variation	Retention time (RT)	% RSD	Response (AU)	% RSD	Recovery (%)
Mobile phase (v/v)	90:10	92:8	5.24	0.65	6952334.33	0.16	99.01
		88:12	5.23	0.40	6964648.33	0.17	98.63
Flow rate (ml/min)	1	1.2	5.26	0.24	6953973.17	0.12	98.80
		0.8	5.29	0.49	6961096.78	0.20	100.01
Wavelength (nm)	254	256	5.23	0.23	6952442.64	0.16	97.25
		252	5.23	0.91	6945573.43	0.29	99.01
Column temperature (°C)	25	27	5.26	0.56	6947399.61	0.27	98.24
		23	5.28	0.82	6964112.08	0.40	99.88
pH	7.0	7.2	5.22	0.67	6945765.28	0.32	98.20
		6.8	5.24	0.34	6942432.37	0.31	98.99

Table 5: Ruggedness of the method (*n* = 3)

Amount (µg/ml)	Retention time (RT)	% RSD	Response (AU)	% RSD	Recovery (%)
200	5.23	0.66	6948299.57	0.12	99.32
400	5.23	0.65	13439561.34	0.36	99.24
600	5.26	0.25	20674502.25	0.28	99.32
800	5.25	0.49	29626692.80	0.45	99.94
1000	5.27	0.45	41006292.76	0.13	99.42
Extract	5.26	0.53	685483.27	0.16	98.56

CONCLUSION

In conclusion, the proposed method is very suitable and alternative method for assaying the puerarin in routine quality control analysis of PTT extract and formulations because of the excellent linearity, specificity, accuracy and precision.

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